The Synthesis of Peptides by Means of Proteolytic Enzymes

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Nagarse, papain, pepsin and thermolysin were found to catalyze the peptide bond formation between two amino acids or peptides, one protected with a suitable group at the amino group and the other at the carboxyl group. Experiments with various combinations of amino acids and dipeptides show specificity in the catalytic action in varying degree which depending on the nature of the enzyme. Since the condensation takes place efficiently under mild conditions, this method opens up a useful way for the synthesis of peptides.

During the last three decades several important general methods and a large number of coupling reagents for peptide bond formation have been developed.[1] Almost all the coupling methods involve the possibility of side reactions and racemization. Racemization may always be expected to some extent when the carboxyl group of an N-protected amino acid or peptide is activated. Careful control of coupling conditions may be used to minimize or eliminate these side reactions and racemization in chemical synthesis.

On the other hand, proteolytic enzyme is expected to provide a potential method for the peptide synthesis without racemization, if hydrolytic action is supressed.

However, no successful result seems to have been reported except for that of Bergmann and Fraenkel-Conrat which involves the condensation of acylamino acid and amino acid anilide in the presence of papain.[2] Fruton[3] referred to their method as an interesting approach to the synthesis of peptide but one involving difficulties which prevent it from being a general synthetic method.

With the expectation that the above difficulties can be overcome by means of the protecting groups of the substrates, we have examined the possibility of using proteolytic enzymes for practical preparation of peptides since a few years ago. The study has produced a considerable amount of useful findings, and in this paper we give some typical examples from the results obtained so far.

Examination was made of the catalytic action of four representative enzymes[4-7] each from seryl-, thiol-, acid-, and metallo-proteinase groups on the following peptide forming reactions between two amino acids or dipeptides suitably protected at the amino group (carboxyl component) or at the carboxyl group (amine component):

1. Z-X-OH + H-Phe-Val-OBu^t ⟶ Z-X-Phe-Val-OBu^t
2. Z-Phe-X-OH + H-Phe-Val-OBu^t ⟶ Z-Phe-X-Phe-Val-OBu^t
3. Z-X-Y-OH + H-Phe-ODPM ⟶ Z-X-Y-Phe-ODPM
4. Z-X-OH + H-Val-ODPM ⟶ Z-X-Val-ODPM
5. Z-X-OH + H-Phe-Phe-OBu^t ⟶ Z-X-Phe-Phe-OBu^t

In these formulas, X and Y stand for arbitrary amino acid residues with Z, benzyloxycarbonyl; Bu^t, *t*-butyl; and DPM, diphenylmethyl as protecting groups.

Materials and Methods

Enzymes: Papain (2.1×10^2 units/mg, Midori Juji Co.), Nagarse (5×10^2 units/mg, Nagase Co.), Thermolysin (8.08×10^3 units/mg, Daiwa Kasei Co.) and porcine pepsin (1.9×10^3 units/mg, Sigma Chemical Co., U.S.A.) were used without further purification.

Substrates: All the amino acids used had the L-configuration.

Phenylalanine Diphenylmethyl Ester p-*Toluenesulfonate and Valine Diphenylmethyl Ester* p-*Toluenesulfonate*: These were prepared in the same manner as described for the corresponding benzyl ester.[8]

Phenylalanine Diphenylmethyl Ester p-*Toluenesulfonate*. The yield was 83%, mp 217—220 °C (from methanol–ether).

Found: C, 68.88; H, 5.76; N, 2.71%. Calcd for $C_{29}H_{29}NO_5S$: C, 69.16; H, 5.80; N, 2.78%.

Valine Diphenylmethyl Ester p-*Toluenesulfonate*. The yield was 65%, mp 173 °C (from methanol–ether).

Found: C, 65.41; H, 6.51; N, 2.96%. Caldc for $C_{25}H_{29}NO_5S$: C, 65.91; H, 6.42; N, 3.07%.

The following dipeptide derivatives were prepared by the procedure given in literature: H-Phe-Val-OBu^t,[9] H-Phe-Phe-OBu^t,[10] Z-Phe-Gly-OH,[11] Z-Phe-Ser-OH,[12] Z-Phe-Arg-(NO_2)-OH,[13] Z-Leu-Phe-OH,[14] Z-Phe-Tyr-OH.[15]

Z-Phe-Val-OEt. To 48.5 g (0.267 mol) of H-Val-OEt in 180 ml of DMF at −10 °C was added 30 ml (0.270 mol) of *N*-methylmorpholine, 80 g (0.267 mol) of Z-Phe-OH, 36.5 g (0.27 mol) of 1-hydroxy-benztriazole and 56.7 g (0.276 mol) of dicyclohexylcarbodiimide in 180 ml of of DMF. After 20 h, the reaction mixture was worked up by the usual procedure. The product was recrystallized from ethanol–water: yield 107.5 g (94%); mp 103—105 °C; $[\alpha]_D$ −18.4° (*c* 1, ethanol).

Found: C, 67.45; H, 7.09; N, 6.61%. Calcd for $C_{24}H_{30}N_2O_5$: C, 67.58; H, 7.09; N, 6.57%.

Z-Phe-Val-OH. A solution of 60 g (0.141 mol) of Z-Phe-Val-OEt in 350 ml of ethanol was saponified with 180 ml of 1 M aqueous sodium hydroxide for 12 h. The solution was acidified with 2M hydrochloric acid and filtered. The sample was recrystallized from ethanol–water for analysis: yield 52.4 g (93%); $[\alpha]_D$ −7.1° (*c* 1, ethanol).

Found: C, 66.11; H, 6.60; N, 7.05%. Calcd for $C_{22}H_{26}N_2O_5$: C, 66.31; H, 6.58; N, 7.03%.

General procedures of the synthesis with enzymes are as follows.

Peptide Synthesis by Papain: 20 ml of a McIlvaine buffer solution having pH 6.6 was added to 1.0 mmol each of a carboxyl component and an amine component. 150 mg

of papain and 0.1 ml of 2-mercaptoethanol were then added to the mixture. After incubation at 38° for 24 h, the resulting precipitate was filtered off and washed in succession with water, 7% aqueous ammonia, 0.5 M hydrochloric acid and water. The product was dissolved in 50 ml of hot methanol and the solution was treated with active carbon. The solution was concentrated *in vacuo* and the residue was recrystallized from an appropriate solvent to give a pure crystalline product.

Peptide Synthesis by Nagarse: 20 ml of a McIlvaine buffer solution having pH of 7.2 was added to 1.0 mmol each of a carboxyl component and an amine component. 150 mg of nagarse was then added to the mixture. After incubation at 38 °C for 24 h, the product was isolated by the same procedure as described above.

Peptide Synthesis by Pepsin: 20 ml of an acetate buffer solution having pH of 4.5 was added to 1.0 mmol each of a carboxyl component and an amine component. 100 mg of pepsin was then added to the mixture and the mixture incubated at 38 °C for 24 h. The product was isolated by the same procedure as described above.

Peptide Synthesis by Thermolysin: 20 ml of a veronal buffer solution having pH of 7.5 was added to 1.0 mmol each of a carboxyl component and an amine component. 20 mg of thermolysin was then added to the mixture. After incubation at 38 °C for 24 h, the product was isolated by the same procedure as described above.

Results and Discussion

The effect of papain and thermolysin on the reaction of benzyloxycarbonyl amino acids with phenylalanyl-valine *t*-butyl ester is given in Table 1.

Table 1. Effect of enzymes on th ereaction
Z–X–OH+H–Phe–Val–OBu^t→Z–X–Phe–Val–OBu^t

Compound(–X–)	Enzyme Yield (%)	
	Papain	Thermolysin
Gly	43	85
Val	—	47
Tyr	15	63
Ser	31	76
Arg(N_{O_2})	49	58

While papain and thermolysin displayed a similar synthetic activity toward these substrates, nagarse and pepsin were ineffective in reaction 1. Thermolysin appears to be more potent than papain in the tripeptide formation.

The effect of papain, thermolysin and nagarse on the reaction of Z–Phe–X–OH with phenylalanyl-valine *t*-butyl ester is given in Table 2.

Table 2. Effect of enzymes on the reaction
Z–Phe–X–OH^t+H–Phe–Val–OBu^t→Z–Phe–X–Phe–Val–OBu^t

Compound(–X–)	Yield (%)		
	Papain	Thermolysin	Nagarse
Gly	77	79	—
Val	12	50	22
Tyr	—	72	35
Ser	84	65	—
Arg(NO_2)	91	52	21

Papain, thermolysin and nagarse exhibit a synthetic activity, while pepsin was ineffective in these tetrapeptide formations.

A comparison of the results obtained in reactions 1 and 2 shows that acyl dipeptides (Z–Phe–X–OH) are more prone to give condensation products than acyl amino acids (Z–X–OH) as carboxyl component.

The effect of papain and pepsin on the reaction of benzyloxycarbonyl dipeptide acids (Z–X–Y–OH) with phenylalanine diphenylmethyl ester is given in Table 3.

Table 3. Effect of enzymes on the reaction
Z–X–Y–OH+H–Phe–ODPM→Z–X–Y–Phe–ODPM

Compound (–X–Y–)	Yield (%)	
	Papain	Pepsin
Leu–Phe	98	84.7
Phe–Tyr	94	63
Val–Tyr	100	0

Both enzymes show the same synthetic activity in the case of Z–Leu–Phe–OH or Z–Phe–Tyr–OH with phenylalanine diphenylmethyl ester. It is of interest that there is a striking difference between these enzymes in the case of Z–Val–Tyr–OH as carboxyl component. However, nagarse and thermolysin were ineffective in these reactions.

The effect of papain on the reaction of benzyloxycarbonyl amino acids with valine diphenylmethyl ester is given in Table 4.

Table 4. Effect of papain on the reaction
Z–X–OH+H–Val–ODPM→Z–X–Val–ODPM

Compound (X)	Yield (%)	Compound (X)	Yield (%)
Ala	80	Phe	91
Met	81	Glu	61
Thr	66	Asn	25
Gln	69	Arg(NO_2)	83
Lys(z)	70		

Table 5. Effect of papain and thermolysin on the following reaction
Z–X–OH+H–Phe–Phe–OBu^t→Z–X–Phe–Phe–OBu^t

Compound (–X–)	Yield (%)	
	Papain	Thermolysin
Gly	20	68
Ala	80	61
Leu	32	79
Phe	—	28
Tyr	—	50
Ser	8	—
Thr	71	28
Met	95	74
Cys(Bzl)	69	7
Asn	48	89
Glu	52	79
Gln	78	79

Table 6. Physical properties and elemental analysis of the enzymic reaction products

X	Mp(°C)	$[\alpha]_D$	Formula	Calcd (%) (Found) C	H	N	S
1. Z–X–Phe–Val–OBu^t							
Gly	foam	−18.3 (*c* 1, MeOH)	$C_{28}H_{37}N_3O_6$	65.73 (65.75	7.29 7.45	8.21 8.52)	
Val	178	−45.5 (*c* 1, MeOH)	$C_{31}H_{43}N_3O_6$	67.24 (66.98	7.83 7.73	7.59 7.55)	
Tyr	85	−33.4 (*c* 1, MeOH)	$C_{35}H_{43}N_3O_7$	68.05 (67.98	7.02 7.14	6.80 6.91)	
Ser	135	−34.9 (*c* 1, MeOH)	$C_{29}H_{39}N_3O_7$	64.31 (64.00	7.26 7.33	7.76 7.87)	
Arg(NO_2)	125	−24.9 (*c* 1, MeOH)	$C_{32}H_{45}N_7O_8$	58.61 (58.88	6.92 6.98	14.95 14.53)	
2. Z–Phe–X–Phe–Val–OBu^t							
Gly	85—92	−19.9 (*c* 1, MeOH)	$C_{37}H_{46}N_4O_7$	67.46 (67.52	7.04 6.93	8.50 8.58)	
Val	216	−44.8 (*c* 1, MeOH)	$C_{40}H_{52}N_4O_7$	68.55 (68.52	7.48 7.50	7.99 7.97)	
Tyr	110	−40.6 (*c* 1, MeOH)	$C_{44}H_{52}N_4O_8$	69.09 (68.91	6.85 6.89	7.32 7.56)	
Ser	169	−28.3 (*c* 1, MeOH)	$C_{38}H_{48}N_4O_8 \cdot H_2O$	64.57 (64.46	7.13 6.91	7.93 8.01)	
Arg(NO_2)	115	−29.2 (*c* 1, MeOH)	$C_{41}H_{54}N_8O_9$	61.33 (61.79	6.78 6.65	13.96 13.90)	
3. Z–X–Y–Phe–ODPM							
Leu–Phe	172	−24.5 (*c* 1, DMF)	$C_{45}H_{47}N_3O_6$	74.46 (74.62	6.53 6.67	5.79 5.66)	
Phe–Tyr	165	−23.9 (*c* 0.63, DMF)	$C_{48}H_{45}N_3O_7$	74.30 (74.06	5.85 5.84	5.42 5.35)	
Val–Tyr	196	−34.8 (*c* 0.5, DMF)	$C_{44}H_{45}N_3O_7$	72.61 (72.49	6.23 6.22	5.77 5.84)	
4. Z–X–Val–ODPM							
Ala	92—96		$C_{29}H_{32}N_2O_5$	71.29 (71.28	6.60 6.54	5.73 5.82)	
Phe	106		$C_{35}H_{36}N_2O_5 \cdot 2H_2O$	69.98 (70.21	6.71 6.34	4.66 4.80)	
Thr	89—95		$C_{30}H_{34}N_2O_6$	69.48 (69.19	6.61 6.59	5.40 5.26)	
Met	98		$C_{31}H_{36}N_2O_5S$	67.80 (67.83	6.61 6.60	5.12 5.16	5.84 5.79)
Asn	116—125		$C_{30}H_{33}N_3O_6$	67.78 (68.18	6.26 6.27	7.90 7.41)	
Glu	125—131		$C_{31}H_{34}N_2O_7$	68.12 (68.23	6.27 6.31	5.12 4.84)	
Gln	178—183		$C_{31}H_{35}N_3O_6$	68.24 (67.94	6.47 6.39	7.70 7.74)	
Arg(NO_2)	142		$C_{32}H_{38}N_6O_7$	62.12 (62.48	6.19 6.21	13.58 13.40)	
Lys(z)	117		$C_{40}H_{45}N_3O_7$	70.67 (70.28	6.67 6.66	6.18 6.33)	
5. Z–X–Phe–Phe–OBu^t							
Gly	72	−13.6 (*c* 0.5, MeOH)	$C_{32}H_{37}N_3O_6$	68.67 (68.91	6.66 6.76	7.51 7.58)	
Ala	95	−33.5 (*c* 1, MeOH)	$C_{33}H_{39}N_3O_6$	69.09 (68.95	6.85 6.84	7.32 7.21)	
Leu	96	−32.1 (*c* 1, MeOH)	$C_{36}H_{45}N_3O_6$	70.22 (70.38	7.37 7.57	6.82 6.64)	
Phe	104	−29.5 (*c* 1, MeOH)	$C_{39}H_{43}N_3O_6$	72.09 (71.86	6.67 6.66	6.47 6.39)	
Tyr	119	−26.1 (*c* 1, MeOH)	$C_{39}H_{43}N_3O_7$	70.36 (70.37	6.51 6.38	6.31 6.05)	
Ser	103		$C_{33}H_{39}N_3O_7$	67.21 (67.30	6.67 6.91	7.13 6.88)	

Table 5. Continued

X	Mp(°C)	$[\alpha]_D$	Formula	Calcd (%) (Found) C	H	N	S
Thr	94	−24.9 (*c* 1, MeOH)	$C_{34}H_{41}N_3O_7$	67.64 (67.24	6.85 6.70	6.96 6.88)	
Met	106	−31.3 (*c* 1, MeOH)	$C_{35}H_{43}N_3O_6S$	66.32 (66.30	6.84 6.77	6.63 6.53)	
Cys(Bzl)	68	−35.0 (*c* 1, MeOH)	$C_{40}H_{45}N_3O_6S$	69.04 (68.92	6.52 6.48	6.04 6.06)	
Glu	147—152	−17.6 (*c* 1, DMF)	$C_{35}H_{41}N_3O_8$	66.54 (66.54	6.54 6.41	6.65 6.59)	
Asn	185	−29.2 (*c* 1, DMF)	$C_{34}H_{40}N_4O_7$	66.21 (66.22	6.54 6.52	9.09 8.87)	
Gln	188	−18.3 (*c* 1, DMF)	$C_{35}H_{42}N_4O_7$	66.65 (66.51	6.71 6.71	8.88 8.74)	

Papain exhibits wide peptide formation activity for various amino acids as a carboxyl component.

The effects of papain and thermolysin in the reaction of benzyloxycarbonyl amino acids with phenylalanyl-phenylalanine *t*-butyl ester are compared in Table 5.

These two enzymes show similar activities, the specificity as regards the nature of reacting amino acids being low.

It appears that the low specificity in the peptide bond formation is generally associated with the low specificity in the hydrolysis of protein, such as papain[5] and thermolysin.[7]

The results shown that the utilization of proteolytic enzymes as a catalyst in the formation of a peptide linkage is advantageous for practical synthesis of oligopeptides because of ready condensation under mild conditions.

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